Ph.D. thesis synopsis

Macrophage Subset Depletion as a Strategy to Inhibit Antibiotic-Induced Macrophage Polarisation

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Abstract

Antibiotics have non-antibacterial effects ranging from mitochondrial-dysfunction to immunomodulation. Antibiotic therapy is imperative for treating bacterial infections but antibiotics have off-target effects on host immune cells. These effects could either be contextually beneficial or harmful and could potentially aid generation of infection persistence and antimicrobial resistance (AMR) via host immunosuppression. This thesis explores and reports the immunosuppressive mechanisms of azithromycin (AZM)-induced bacterial persistence in Balb/c model of *Shigella flexneri* infection and increased Colony Stimulating Factor-1 Receptor (CSF-1R) expression on murine macrophages. This work presents the molecular mechanism behind these observations and explores the GW2580-mediated CSF-1R blockade during *S. flexneri* induced infection along with azithromycin treatment to moderate azithromycin-mediated off-target effects on host immune cells. In this study the effect of azithromycin on effector proteins or transcription factors that are involved in macrophage activation as well as are downstream to CSF-1R (ERK1/2, P38, AKT1, STAT3, STAT6, and EGR2) has been investigated. The work partially co-relates the azithromycin induced change in the phosphorylation or expression of these effector proteins with CSF-1R signalling and uses CSF-1R blocker GW2580 along with azithromycin treatment. The validation of *in vitro* results has been done in Balb/c model of *S. flexneri* infection and the effect of GW2580 usage with azithromycin treatment on disease and immunological outcome of *S. flexneri* induced infection has been reported.

INTRODUCTION

This study explores the immuno-modulatory effects of a bacteriostatic antibiotic azithromycin within and outside the context of an intracellular infection caused by enteric diarrhoea causing bacteria *Shigella flexneri.* The study is focused to reconnoitre the effects of azithromycin on macrophage generation, activation, functions and subsequent macrophage mediated T cell responses in presence or absence of *Shigella flexneri* infection.

Antibiotics are prescribed as a primary treatment regimen for bacterial infections. Azithromycin a macrolide antibiotic is a drug of choice for treating diarrhoeal infections caused by invasive bacterial pathogens like *Shigella flexneri* whereas the antibiotics like levofloxacin, ciprofloxacin, rifaximin etc., are generally prescribe for infections caused by non-invasive bacterial pathogens etc., (Tribble, 2017; Williams and Berkley, 2018). However besides their antibacterial activities antibiotics are known to demonstrate immune system alterations/modulations (Ianaro *et al.*, 2000; Fehervari, 2018; Marchant, 2018; Yadav, Shah, *et al.*, 2023).

Generally, during initial phases of an infection particularly for invasive or intracellular pathogens an inflammatory response is required to clear off the infection which should be balanced later on by an anti-inflammatory response leading to tissue healing and homeostasis. This inflammatory response during initial phases of infection is generated by pro-inflammatory macrophages known as M1 macrophages.

On the other hand, the anti-inflammatory response is generally generated during resolving phases of infection by the alternatively active, anti-inflammatory macrophages known as M2 macrophages (Wynn, Chawla and Pollard, 2013; Lim and Song, 2014; Yadav *et al.*, 2022). Macrophages are the innate immune cells known for phagocytic ability and when there is a pathogen encounter in our body the macrophages engulf them and carry out endosomal degradation. Depending on the environmental cues, other cellular and molecular alterations macrophages demonstrate differential phagocytosis and polarisation (Wynn, Chawla and Pollard, 2013).

Owing to the immunomodulatory nature of the antibiotics like azithromycin (Murphy *et al.*, 2008; Feola *et al.*, 2010; Xue *et al.*, 2017), they may directly or indirectly skew macrophages towards the generation of anti-inflammatory M2-like macrophage subset. This may lead to compromised inflammatory M1-like macrophage functions. The M2 subset will drive

generation of T **h**elper (h) 17/Th2/T regulatory (reg) which are the known negative regulators of Th1 (required for clearance of intracellular bacteria) (Mills, 2012; Brambilla *et al.*, 2014; Wang, Liang and Zen, 2014; Yadav *et al.*, 2022). Thus the response would be focused on extracellular clearance and owing to poor Th1 activation may lead to intracellular bacterial persistence which may later on cause secondary infections as seen during *Chlamydia* or *Mycobacterium Tuberculosis* infections.

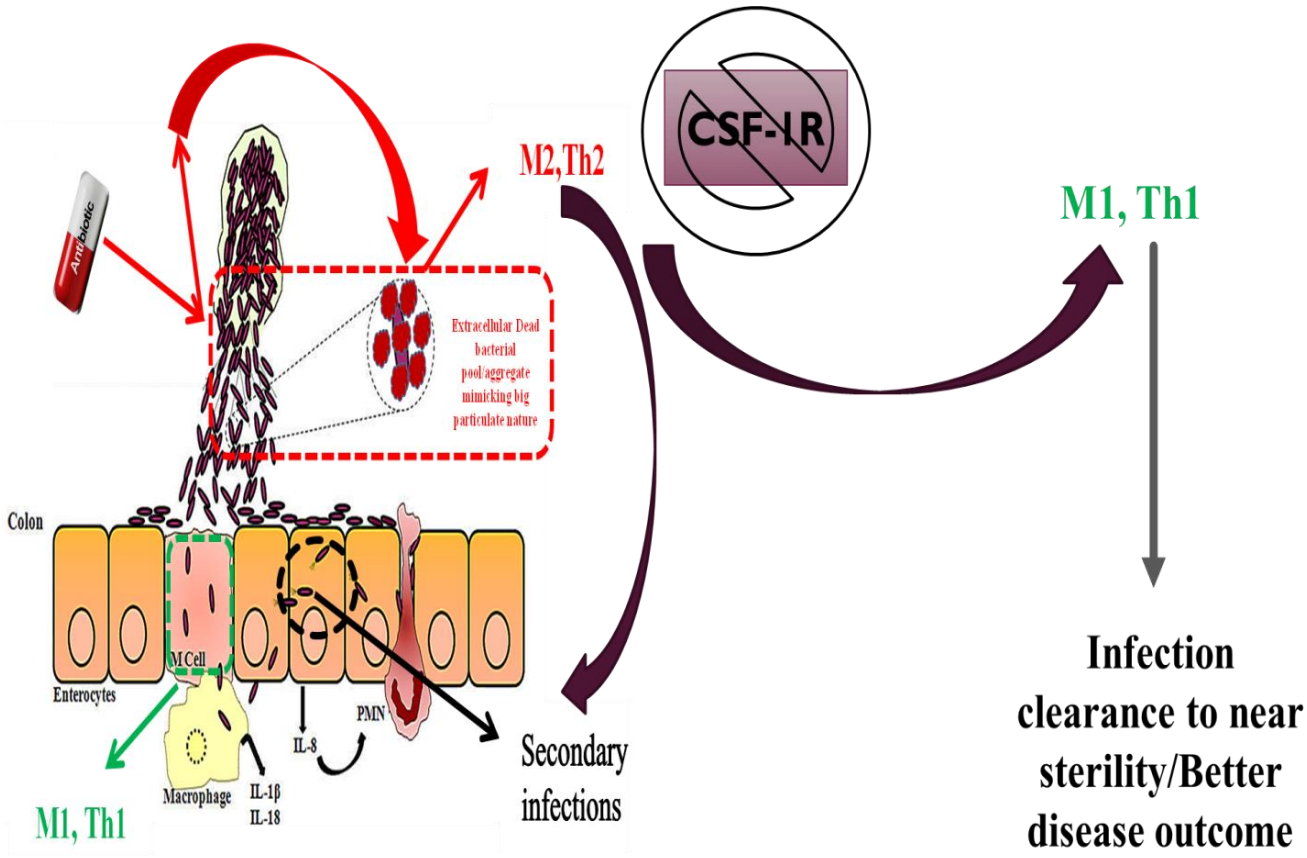
During the course of infection when an antibiotic is taken, the antibiotic will kill the bacteria faster compared to the host immune system generating dead bacterial pools, which might be inefficient in mounting an appropriate immune response against the infection (Cheers and Zhan, 1996; Hirayama, Iida and Nakase, 2017; Ugolini and Sander, 2019). Other than that azithromycin is known to potentiate M2-like macrophage subset could directly alter the immune-response against an infection to anti-inflammatory type irrespective of the nature of the pathogen (Haydar *et al.*, 2019).

Thus firstly, the effect of azithromycin on the macrophage differentiation, activation and functions during *S. flexneri* infection has been explored in this work. Then the effect of azithromycin induced altered macrophage phenotype and macrophage-mediated-T helper response during infection has been investigated. Finally, this thesis work selectively probes into the scope of depleting azithromycin-induced M2-like subsets of macrophages via blocking the **C**olony **S**timulating **F**actor-1**R**eceptor (CSF-1R) that is exclusively found on monocytes and macrophages, and plays role in macrophage differentiation and activation (Hume and MacDonald, 2012; Boulakirba *et al.*, 2018).

The CSF-1R blockade would lead to potentiated M1-mediated Th1 activation and expansion in response to infection despite the immuno-modulatory nature of azithromycin. Thus CSF-1R blockade would result in efficient clearance of intracellular bacteria dodging any scope of asymptomatic, persistent, prolonged or re-current infections due to inefficient bacterial clearance.

HYPOTHESIS

We hypothesised that azithromycin may directly and indirectly skew macrophage generation to M2-subset of macrophages leading to compromised M1 functions and the generation of Th17/Th2/Treg instead of required Th1 during an intracellular infection could occur. Th17/Th2/Tregs are the known negative regulators of Th1 (required for clearance of intracellular bacteria). The response would be focused on extracellular clearance and due to poor Th1 activation may lead to intracellular bacterial persistence. Thus, we aim to selectively deplete M2-subsets via CSF-1R blockade to potentiate M1-mediated Th1 activation.



Graphical representation of hypothesis

OBJECTIVES:

I. To test the effect of antibiotics on macrophage's phagocytic ability, polarization (M1/M2) and inflammasome activation during *Shigella flexneri* infection.

II. Unravelling the effect of antibiotics on *Shigella*-specific T cell activation

III. To evaluate the potential of M2 depletion in an *in vitro* and mouse model of *Shigella flexneri* infection.

MATERIALS AND METHODS

Growth and maintenance of bacteria: *Shigella flexneri* (MTCC 1457) procured from **M**icrobial **T**ype **C**ulture **C**ollection (MTCC), IMTECH, Chandigarh, India, was grown in **L**uria **B**ertani (LB) broth (Himedia, M1245-500G), at 37˚C, 140 **r**evolution **p**er **mi**nute (rpm) in an orbital shaker incubator (Orbitek, Scigenic Biotech, India). The bacteria were maintained on SS agar (Himedia, M-108-500G). For infection an overnight grown, metabolically active culture was used keeping the MOI to 50:1 (50 bacteria per macrophage) for all experiments unless otherwise mentioned (Yadav, Dalai, *et al.*, 2023).

Mice: 25-40g healthy Balb/c male mice housed separately in BV Patel PERD centre, Thaltej, Ahmedabad and/or Prerana Bio-Innovations Research Pvt. Ltd, Gandhinagar, animal house facility in an air-conditioned room at 25˚C with autoclaved food and water *ad libitum* (Yadav, Dalai, *et al.*, 2023).

***S. flexneri* for infection:** Metabolically active *S. flexneri* were used for infection studies. Every time an overnight-grown bacterial culture was used after adjusting to the required CFU by measuring OD_{595}. Animals were infected via **i**ntra**p**eritoneal (i.p.) route for establishing infection using 1 ml syringe in **P**hosphate **B**uffer **S**aline (PBS) keeping the injection volume to 200 µl, everything was done in sterile conditions in the **B**io-**S**afety **L**evel (BSL)-II facility (Yadav, Dalai, *et al.*, 2023).

Azithromycin and GW2580 treatment: Azithromycin (100mg/kg body weight) and GW2580 (50mM/kg body weight) were injected via the i.p. route using 1ml syringe (Yadav, Dalai, *et al.*, 2023).

Generation of Bone Marrow Derived Macrophages (BMDMs): BMDMs were generated following an already established lab protocol (Patel *et al.*, 2018; Shah *et al.*, 2021; Yadav,

Dalai, *et al.*, 2023). Briefly, for generating M1-like Mφs media supplemented with GM-CSF (20 ng/ml), IFNγ (10 ng/ml) and/or LPS (1μg/ml) was used. For generation of M2-like Mφs media supplemented with MCSF (40ng/ml), IL-4 (10 ng/ml) with or without IL-10 (10 ng/ml) supplemented media was used. Wherever required, AZM 50 μg/ml was added. The cells were plated on day 1 with MCSF or GMCSF, and media change was done on days 3 and 5. The treatments were given on days 5, 6, or 7 according to the designed experiment. M1 or M2 polarising signals were added on day 5 in most of the experiments. The cells were cultured in RPMI 1640 media supplemented with 1x sodium pyruvate and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Yadav, Dalai, *et al.*, 2023).

Faecal and Splenic Bacterial count: Faeces were collected day-wise and weight was normalised for each group. The Faeces were then resuspended in **P**hosphate **B**uffered **S**aline (PBS) and kept shaking at 37˚C and 100 rpm, overnight. The next day, the faecal bacterial count was measured using the drop plate method on SS agar. Similarly, the spleen was cut to an equal weight for each group and minced in PBS; the bacterial count was done using the drop plate method on SS agar (Yadav, Dalai, *et al.*, 2023).

Visible disease parameter measurement: Body weight was measured daily on a weighing machine provided by the BV Patel PERD Centre or Prerana Bio-Innovations Research Private. Limited, and surface abdominal body temperature was measured using a digital clinical thermometer. All the other visible disease symptoms: Ruffled skin, loss of activity, abdominal swelling, soiled anus, conjunctivitis, body shivering, loss of appetite; diarrheal faeces were scored via the blind scoring method and were recorded manually (Yadav, Dalai, *et al.*, 2023).

Tissue cytokine measurement

Splenic cytokines: Spleen from each group was collected in RPMI media and minced in 1 ml of RPMI media in sterile conditions. The supernatant was collected after centrifugation and stored at -20˚C. The cytokine was measured using the lab-optimised **E**nzyme-**L**inked **I**mmunosorbent **A**ssay (ELISA) protocol (Yadav, Dalai, *et al.*, 2023).

Serum cytokines: Serum cytokines from each group were measured using the lab ELISA protocol. Blood from the retro-orbital plexus was collected on the day of sacrifice and serum was isolated by centrifuging blood at @3000 rpm for 10 minutes. The upper layer of serum

was stored at -20˚C until ELISA was performed for the required cytokines (Yadav, Dalai, *et al.*, 2023).

Intestinal cytokines: The intestine was minced as described above, and the supernatant was collected and stored at -20˚C until ELISA was performed for the required cytokines (Yadav, Dalai, *et al.*, 2023).

Lymph node cytokines: Lymph nodes from each group were collected in RPMI media and minced in 1 ml of RPMI media in sterile conditions. The supernatant was collected after centrifugation and stored at -20˚C. The cytokines were measured using lab optimised ELISA protocol (Yadav, Dalai, *et al.*, 2023).

T cell re-challenge assay cytokines: Lymph nodes from each group were and minced in 1 ml of RPMI media in sterile conditions, centrifuged at 850g, 5 min, 4˚C and total T cells were collected. Mφs were already activated for 12-18hrs by incubation with bacteria or LPS, and then total T cells were added in a ratio of 1:10 and incubated for 72 hrs. Only Mφs/only T cells were used as normalising controls. Afterward, cells were collected for flow cytometry, and the supernatant was collected for ELISA. The ELISA graphs were plotted after normalising the values with controls (Yadav, Dalai, *et al.*, 2023).

Bromodeoxyuridine or 5-bromo-2'-deoxyuridine (BrdU) incorporation assay: Lymph nodes from each group were collected in RPMI media and minced in 1 ml of RPMI media in sterile conditions, centrifuged at 850g for 5 min, at 4˚C and total T cells were collected. For direct- BrdU at a +concentration of 3µg/ml was added and cells were incubated for 24 hrs, stained with anti-BrdU labelled with **F**luorescein **i**so**t**hio**c**yanate (FITC), and for Mφs were already activated for 12-18hrs by incubation with bacteria or LPS, and then total T cells were added in a ratio of 1:10 along with BrdU, incubated for 72 hrs, and stained with anti-BrdU labeled with FITC (Yadav, Dalai, *et al.*, 2023).

Carboxyfluorescein Succinimidyl Ester (CFSE) T cell proliferation assay: CFSE is a permeable dye, which is non-fluorescent till it enters live cells and binds amine groups via succinimidyl group. Inside cells the esterase enzymes hydrolyse the ester to release fluorescent amine-reactive dye. Thus, CFSE could be used to monitor distinct generations of proliferating cells via dye dilution method. After each multiplication the cells appear as a distinct peak in a flowcytometry histogram. Total T cells from lymph nodes of each group were isolated as described above and centrifuged to obtain cell pellet. The cell pellet was

resuspended gently in PBS containing CFSE (5μM). The cells were incubated for 20 minutes at 37°C for labelling. Labelled cells were pelleted down and resuspended in fresh culture medium. After the required treatment and incubation, cells were collected, fixed and stained for CD3 marker and flowcytometric analysis was done (Yadav, Dalai, *et al.*, 2023).

Flow cytometry: Cells were collected and stained for flow cytometry analysis. For intracellular staining, brefeldin A solution in DMSO had been added 12 hrs before collection at a concentration of 1 μg/ml. Positive control T cells were activated with CD28/3 or concavalin A. Staining with CD11b, CSF-1R, CD68, CD86, CD206, CD4, CD8, FOXP3, T-bet, IFN-γ, IL-10, CD11b, F4/80, ERK, pERK, STAT3, pSTAT3, STAT6, pSTAT6, AKT1, EGR2, CSF-1R, and TGF-β (500x dilution each) was done using lab optimised protocol. Cells were blocked with Fc block CD16/32 for 30min before staining as a background staining control. Everything was done on ice. The acquisition was done on Beckman Coulter, Cytoflex research flow cytometer. Data were analysed using CytExpert 2.4 from Beckman Coulter (Yadav, Dalai, *et al.*, 2023).

Protein expression: Briefly, **R**adio **I**mmuno **P**recipitation **A**ssay (RIPA) buffer supplemented with **P**henyl**m**ethyl**s**ulphonyl **F**luoride (PMSF) and **P**rotease **I**nhibitor (PI) according to the manufacture's recommended concentration was used to lyse cells. After half an hour of incubation on ice with intermittent vortexing, samples were centrifuged at 10,000 rpm for 15 min, the supernatant was collected. Bradford's assay was done, each sample was normalised to 50μg/40μl protein and SDS-PAGE was run followed by Western blotting for ASC, NLRP3, Caspase-1, ERK, pERK, P38, pP38, pCSF-1R and GAPDH. NLRP3 was run on 4% stacking, 10% resolving gel, ASC, and Caspase1 were run on 4% stacking, 14% resolving gel. 4% stacking and 12% and 8% resolving gels followed by Western blotting were used for ERK, pERK, P38, pP38, pCSF-1R. Primary antibody dilution used was 1:1000 and secondary antibody dilution used was 1:5000 (Yadav, Dalai, *et al.*, 2023).

Nitric Oxide assay (NO): NO assay was done using Griess Reagent according to the manufacturer's protocol. The assay determines nitric oxide concentrations based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. Nitrite is further metabolised to **R**eactive **N**itrogen **S**pecies (RNS) i.e., nitric oxide. RNS (NO) is a functional output of iNOS and is produced more by M1-like Mφs and causes the oxidative stress-mediated killing of the internalised pathogen. Murine Mφs were seeded in a 96-well plate, at a density

of 10^4 cells/ well, and incubated at 37˚C, 5% CO_2 overnight in colorless DMEM. The LPS/IFN-γ, AZM treatment was given and incubated for 12-18 hrs, at 37˚C, 5% CO_2. The supernatant was collected and used for estimating the released NO levels as a measure of iNOS activity (Yadav, Dalai, *et al.*, 2023).

Arginase activity: Arginase activity was measured using a lab-optimised protocol (Shah *et al.*, 2021). Briefly, murine Mφs were seeded in a 6-well plate at a density of 10^6 cells/ well, and incubated at 37˚C, 5% CO_2 overnight. The IFN-γ 10ng/ml, AZM 50 µg/ml, LPS at 1 µg/ml, and infection at MOI of 50:1, treatment was given and incubated for 24 hrs at 37˚C, 5% CO_2. The protein was extracted in a tris-Cl buffer with PMSF and PI according to the manufacturer's recommended concentration. After the solution was centrifuged at 10,000 rpm for 15 min (Eppendorf, 5804 R), the supernatant was collected. Protein concentration was estimated per sample using lab-optimised Bradford's assay: each sample was normalised to 20µg/20µl protein, and arginase activity was measured (Yadav *et al.*, 2023).

Caspase-3 activity: Caspase-3 activity was measured using lab optimised protocol. Briefly, murine Mφs were seeded in a 6-well plate, at a density of 10^6 cells/ well, and incubated at 37˚C, 5% CO_2 overnight. AZM 50 µg/ml, LPS 1 µg/ml, infection at an MOI of 50:1 were added to the cells and incubated for 24 hrs at 37˚C, with 5% CO2. The protein was extracted in a tris-Cl buffer with PMSF and PI according to the manufactures recommended concentration. After the solution was centrifuged at 10,000 rpm for 15 min, the supernatant was collected. Bradford's assay was done, and each sample was normalised to 50µg/80µl protein, and caspase3 activity was measured. Briefly, 20µM Caspase3 substrate (sigma SCP0084) was added and incubated at 37˚C for half an hour, then 10µl of stop solution was added, and OD was taken at 415nm. Caspase-3, a cysteine protease activator of caspase-6 and caspase-7, metabolises the amyloid-β 4A precursor protein. We have detected Caspase-3 using chromogenic peptide substrates built around the DxxD (asp-x-x-asp) sequences DMQD and DEVD-like chromogenic; Ac-DMQD-AMC (Acetyl-Asp-Met-Gln-Asp- amino-4-methyl coumarin), which gives blue color after cleavage (Yadav *et al.*, 2023).

Phagocytic assay: 10^5 BMDMs were generated in a 48-well plate, incubated at 37ºC, with 5% CO_2. *S. flexneri* MOI 50:1, prepared in RPMI media (antibiotics, serum-free) was used for infection. Cells were given AZM treatment pre- or post-infection, and incubated for 1 hour at 37ºC. Medium was removed from the plate and gentamicin + fresh media was added to each well, incubated for 15/ 30/ 160 minutes (adhesion assay, phagocytic assay, and

killing assay respectively) at 37ºC, lysis was done with 0.1% TitronX-100. The lysate was collected in tubes and 10^{-3} dilution was spread on LB plates and incubated overnight at 37ºC, and colonies were counted. For imaging, the bacteria were stained with EtBr (10 µg/ml) in PBS, kept for 15 min at incubation, given cold shock at 4˚C for 20 min, washed to remove the extra stain, and added to the murine Mφs for assessing phagocytosis via immunofluorescence (Yadav *et al.*, 2023).

RNA extraction and cDNA preparation: 10^6 cells were seeded to generate BMDMs as described above in a 12-well plate and were given AZM (50 µg/ml), IFN-γ 20ng/ml, IL-4 (10ng/ml), and IL-10 (10 ng/ml) treatments. RNA was extracted using the TRIzol method, and cDNA was prepared using a BioRad kit. Real-time PCR was done using the Applied Biosystems Real-Time PCR machine (Yadav *et al.*, 2023).

Cytokine measurement in culture supernatant: BMDMs were generated as described above. Wherever required, AZM 50µg/ml was used either with the M1 stimulus or after polarising to M1 and without any stimulus, with or without the CSF-1R blocker (GW2580). The supernatant was collected and measured for cytokines using lab-based ELISA protocol (Yadav *et al.*, 2023).

T cell proliferation assay:

T cell isolation: Total T cells were isolated using a sterile nylon wool column. The spleens of healthy 4-6 weeks old Balb/C male mice were taken. The spleens were minced and cells were collected in a fresh microfuge tube, in RPMI media, collected cells were passed through the nylon column, which was pre-equilibrated with media. The run-through after passing the cells was collected and the process was repeated 2 to 4 times until the maximum cells were retrieved. The B cells attach to the nylon wool, and the total T cells ($CD8^+$ and $CD4^+$) were collected in the run-through and counted for further experiments (Yadav *et al.*, 2023).

Macrophage-T cell co-culture: T cell co-culture assay was done using the lab-established protocol. Briefly, BMDMs were generated in a 96-well, U-bottom plate following lab protocol. After the generation of BMDMs, the required treatment was given. Total T cells were added at a ratio of 10:1 and incubated for 48-72 hrs. The supernatant was collected to measure cytokine production, and cells were subjected to an MTT assay to measure cellular proliferation (Yadav *et al.*, 2023).

siRNA transfection assay: Anti-CSF-1R siRNA along with a negative control was used to transfect AZM-treated RAW264.7 cells (MCSF treated) using lipofectamine 2000 reagent. Cells were pre-treated with AZM (50µg/ml) overnight in antibiotic-free, reduced serum opti-MEM. After transfection cells were incubated for 18 hrs with media change at 5-6 hrs, after which cells were collected and stained for CSF-1R and flow cytometry was done (Yadav *et al.*, 2023).

Percentage Mean fluorescence Intensity: Mean **F**luorescence **I**ntensity (MFI) was calculated - The whole field was divided into four equal parts: ImageJ> Randomly selected a cell/part using any of the drawing/selection tools analyze x 10> set measurements > area integrated intensity and mean grey value selected > Measured> popup box with values for that cell> selected a region next to the cell that had no fluorescence for background subtraction> Repeated> selected all data in the Results window> copied and pasted into spreadsheet> corrected total cell fluorescence (CTCF)= Integrated Density – (Area of selected cell X Mean fluorescence of background readings)> plotted using GraphPad Prism 7 (Yadav *et al.*, 2023).

Bone-marrow cell isolation for transcriptional analysis: The femurs and tibias from mice hind legs were collected at the time of sacrifice in RPMI media and kept on ice until further processing. The bone-marrow cells were isolated using a lab optimised protocol (Yadav, Dalai, *et al.*, 2023). The isolated cells were treated with Gey's solution to lyse red blood cells and washed with media at least twice to remove any residual Gey's solution. The cells were resuspended in media for counting. The cells were then stored in TriZol keeping the cell count to minimum $2X10^6$/sample, at -20˚C until further processing. The stored samples were further outsourced for mRNAseq analysis.

mRNA sequencing: The RNA sequencing was outsourced from Eurofins India. Three biological replicates were given / sample for RNA sequencing. Global reference-based mRNA sequencing was requested. RNA Seq library was prepared using TruSeq Stranded mRNA Sample Prep Kit and library validation was done using Agilent 4200 Tape Station. Sequencing was done on Illumina NextSeq500 platform. Raw data were obtained as a compressed archive of FASTQ files for each sample.

Transcriptomic analysis: The transcriptomic analysis was done using an online RNA sequencing analysis cloud platform RaNA-seq. RaNA-Seq is an open bioinformatics tool and is good for rapid analysis of RNAseq data. Using RaNA-Seq quality control metrics was

calculated and determined, differential expression analyses and functional analyses (functional enrichment and **g**ene **s**et **e**nrichment (GSEA)) were done using pre-build analysis pipeline. Analysis parameters followed were- method; DEseq2/EdgeR, test; Wald/QLF, pValue cut off-; 0.05, fit type; parametric (Prieto and Barrios, 2020)(Prieto and Barrios, 2020).

Relative Density: Relative **D**ensity (RD) was calculated as: ImageJ> Selection of the largest band> set measurements > area integrated intensity and mean grey value selected > measured> popup box with values for that band> selected a region next to the band that had no intensity for background subtraction> repeated, keeping the selection area same for each band> selected all data in the results window> copied and pasted it into spreadsheet> inverted mean intensity = (255-mean intensity)> corrected total intensity = (inverted mean Intensity of bands – inverted mean intensity of background readings)> normalised with housekeeping gene (β-Actin or GAPDH)> plotted using GraphPad Prism 7.

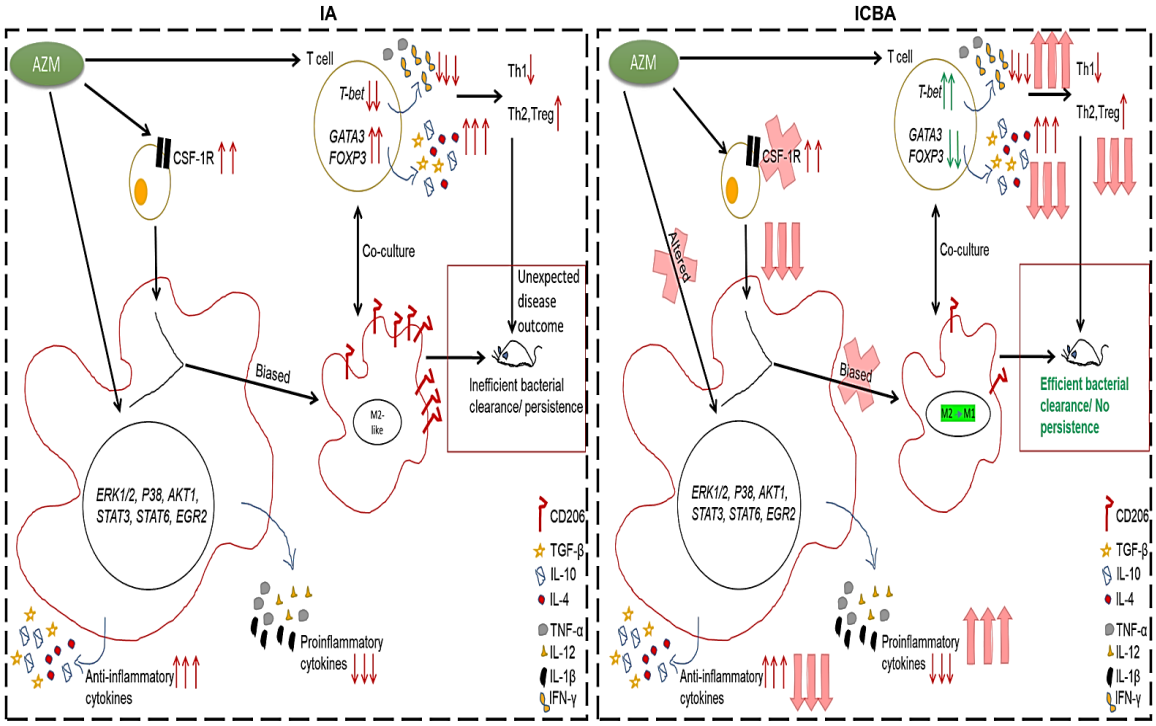
Statistical analysis: All the analyses were done using GraphPad Prism7. Images were processed using ImageJ 2.5. Experiments were repeated at least twice/thrice. One-way **an**alysis **o**f variance (ANOVA) along with Tukey's test with multiple comparisons was used for statistical significance, $p \leq 0.05$ was considered significant.

SUMMARISED RESULTS

Azithromycin altered the macrophage (Mφ)-functions/phenotype, macrophage-mediated T cell responses from required M1-T helper (h) 1 to M2-Th2/T regulatory (reg) during intracellular *S. flexneri infection*. **Immunosuppressive-mechanism:** AZM increased *CD206, CSF-1R, egr2, arg1, IL-10* (M2-marker), reduced *CD68, iNOS, asc, nlrp3, aim2, nlrp1a, caspase1/3, TNF-α, IL-12, IL-1β* expressions/activity/release (M1-marker) by AZM/AZM-treated-infected Mφs(a/aiMφs) indicating that a/aiMφs were primed towards anti-inflammatory-phenotype. CSF-1R-blockade increased *NO, IL-12, TNF-α, IL-1β* and decreased *TGF-β, CD206 r*elease/expression in aiMφs. Decreased *cd40, cd86, cd80* transcripts in a/aiMφs mediated decreased $CD8^{+}/4^{+}$ *T-cell proliferation, T-bet, IFN-γ, IL-2/17*, increased *FOXP3, TGF-β, IL-4* (rescued with CSF-1R blockade). **Molecular-Mechanism:** a/aiMφs had increased *PU.1, C/EBPβ, CSF-1R/pCSF-1R, pERK1/2/ERK1/2, AKT1/STAT6/3* and decreased *pP38/P38* expressions. PU.1, C/EBPβ upstream to *csf-1r* do its transcriptional-regulation and ERK1/2, P38 are downstream to it. ***In vivo:*** Systemic immune-response was shifted to *M2-mediated-TH2/Treg* in AZM-administered-groups resulting in

visibly asymptomatic-disease with splenic-bacterial load, intestinal-length and histopathology-scores, swollen lymph-nodes comparable to infected-mice while *visible disease-scores comparable to uninfected-mice*. CSF-1R-blockade with AZM-treatment resulted in better disease-outcome in mice. We conclude that AZM-treatment during intracellular-bacterial-infections might cause asymptomatic-persistence of a small bacterial-population, not showing any visible disease-symptoms, rendering host at risk of getting secondary/repetitive-infections like observed in cases of *Mycobacterium tuberculosis* infections, etc. However it needs further validation in pre-clinical and clinical set-up. This work gives new insights into the host-pathogen-antibiotic axis of the infection and immune response which could prove to be useful for developing new treatment-strategies against the infections.

This study explored the immuno-modulatory effects of a well-known and widely prescribed bacteriostatic antibiotic azithromycin inside and outside the context of an intracellular infection. The work presented in this thesis established that azithromycin amplified the expressions of various effector proteins like PU.1, C/EBPβ, EGR2 etc., along with augmented CSF-1R/pCSF-1R on macrophages. It was further observed that this lead to the skewing of bone-marrow derived macrophage generation to an anti-inflammatory type. Azithromycin also changed the general macrophage functions like phagocytosis, oxidative stress generation and oxidative stress-mediated-killing of the internalised bacteria. The inflammasome activation that happens in response to the infection or PAMPs was also observed to be hampered or reduced.



The expressions of several inflammasome components like nlrp3, aim2, nod1, nod2, nlrp1a, asc were reduced at transcriptional level in macrophages even in presence of S. flexneri. The translational expressions of ASC, NLRP3 were also reduced along with reduced release of inflammatory mediator IL-1β in azithromycin treated macrophages in response to S. flexneri infection. This indicated that the activation of inflammasome in response to infection was generally hampered in azithromycin treated macrophages. Moreover, the ARG1, CD206 expressions in infected macrophages were increased by azithromycin. The azithromycin-administered mice also showed heightened expressions of CSF-1R, CD206 on splenic macrophages. This indicated that the emergency myelopoiesis in response to infection or bacterial PAMPs might have been shifted to an anti-inflammatory type under the influence of azithromycin.

The spleen is secondary lymphoid organ and thus is a site for secondary myelopoiesis during infections, which is imperative for generation a fitting immune response to get rid of the infection to near sterility. Accordingly, the macrophage-dependent T cell responses were also observed to be altered and hampered in azithromycin administered mice even in presence of infection. The overall helper T cell response was shifted to Th2/Treg instead of required Th1 in both the in vitro and in vivo setting.

Graphical summary of conclusion

It was observed that these immuno-altering effects of azithromycin were reversed when CSF-1R signalling was blocked by using a small molecular inhibitor GW2580 for CSF-1R

blockade on macrophages both in vitro and in vivo. Hence, azithromycin-mediated skewed immune-response to an anti-inflammatory or regulatory phase could be because of altered CSF-1R expression on macrophages, which is known to bias macrophage-generation to M2-like phenotype. Interestingly, the azithromycin-induced anti-inflammatory effects could be beneficial for the host during the resolving phases of the infection that requires dampening of the inflammatory reactions and mediators generated in response to infection. Yet it could lead to an inefficient bacterial clearance and can also aid bacterial persistence or the appearance of secondary infections in the long run. Thus I conclude that this observed shift in the immune-response was mostly prompted by the azithromycin-mediated skewed macrophage functions and phenotype.

This thesis presents a work that explored an inimitable antibiotic-immune-infection axis in the context of an intracellular infection. To further summarise the molecular aspects of these azithromycin-induced alterations the presented work explored the CSF-1R and its downstream signalling effector proteins like ERK1/2, P38, AKT1, EGR2, STAT3, and STAT6. Azithromycin affected the expressions/phosphorylation of all these mentioned transcription factors leading to altered production of downstream proteins like various cytokines by the macrophages. This thesis delivered the physiological and immunological evidences of better infection clearance leading to a better disease outcome with the use of GW2580 (CSF-1R blocker) before azithromycin treatment regimen in S. flexneri infected mice. This was also reflected in the transcriptional analysis, which co-related well with the disease outcome and validated the experimental observations.

Moreover, this approach of CSF-1R blockade could further be developed and tested for increasing the effectiveness of available immunosuppressive antibiotics that pebbledash the threat of resistance during in vivo scenario. Though this study has its own limitations thus it should not be directly adapted to a clinical setup. Yet this study presents a platform for development of better-quality therapeutics to increase the in vivo sensitivity of already available immunosuppressive antibiotics. Therefore, it warrants more research to be done on exploring the CSF-1R signalling axis and efficacy of its blockade to restore M2 to M1 balance, which would facilitate the immuno-permissive environment in immunosuppressive patients who are on long-term antibiotic therapy or in patients on long-term immunosuppressive antibiotics like azithromycin.

This would lead to better bacterial clearance and would eventually limit the extended exposure time of antibiotics to bacteria during the in vivo settings. As already discussed in earlier sections the antibiotics have long been recognised for their immuno-modulatory

actions. So to clear off an infection close to sterility out of the host, not only the antibacterial actions of antibiotics are vital but a positive association with immune activation is also a prerequisite. Also, the inertness of the antibiotics to the immune system of host is essential. More research is required to better understand the effects of long-term azithromycin treatment or abuse. The clinical relevance of azithromycin treatment in patients on long-term antibiotic administrations/immuno-suppressive patients with antibiotic prescriptions should be correlated with its immuno-modulatory affects.

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